keratan sulfate proteo-glycans

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KERATAN SULFATE PROTEOGLYCANS

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Methods for isolation and characterization of proteoglycans.

I) EXTRACTION AND PURIFICATION OF PROTEOGLYCANS FROM VARIOUS TYPES OF CONNECTIVE TISSUE.
Biochim. Biophys. Acta 338 (1974) 108-119
C.A. Antonopoulos, I. Axelsson, D. Heinegård & S. Gardell

II) CHARACTERIZATION OF PROTEINS AND OTHER MACROMOLECULES BY AGAROSE GEL CHROMATOGRAPHY.
I. Axelsson

Bovine corneal proteoglycans: Distribution of keratan sulfate and characterization of keratan sulfate proteoglycans.

III) FRACTIONATION OF PROTEOGLYCANS FROM BOVINE CORNEAL STROMA.
Biochem. J. 145 (1975) 491-500
I. Axelsson & D. Heinegård

IV) CHARACTERIZATION OF KERATAN SULPHATE PROTEOGLYCANS FROM BOVINE CORNEAL STROMA.
I. Axelsson & D. Heinegård

Bovine skeletal proteoglycans: Distribution of keratan sulfate and characterization of keratan sulfate-rich regions in proteoglycans.

V) DISTRIBUTION OF KERATAN SULFATE IN CARTILAGE PROTEOGLYCANS
J. Biol. Chem. 252 (1977) In press
D. Heinegård & I. Axelsson

VI) SKELETAL KERATAN SULFATE PEPTIDES FROM DIFFERENT TISSUES-CHARACTERIZATION AND ALKALI DEGRADATION.
D. Heinegård, I. Axelsson & S. Inerot

PREFACE

The present thesis is a summary of the six papers listed on the preceding page. They are referred to in the text by their Roman numerals.

I am very grateful to my teacher Dr Dick Heinegård who with brilliant ideas and continuous support guided me throughout this investigation and to Annika Björne--Persson whose expert technical assistance made the experimental work possible. Much advice, help and encouragement were obtained from Professor Sven Gardell and the other members of his research group in Lund, from Drs Torvard C. Laurent and Håkan Pertoft, Uppsala, and from Drs David S. Howell, Francisco J. Müller, and Julio C. Pita, Miami. The manuscripts were carefully and patiently typed by Marianne Larsson and Lillemor Hansson. Finally, I wish to thank my wife Ingrid and my family for never--failing support.

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The following abbreviations were used in figures and tables: CS, chondroitin sulfate; Coll., collagen; Fuc, fucose; Gal, galactose, GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; GlcUA, glucuronic acid; HA, hyaluronate; $HexNH_2$, hexosamine; HS, heparan sulfate; KS, keratan sulfate; Man, mannose; NANA, N-acetylneuraminic acid; and Xyl, xylose.

Lund in april, 1977.

Inge Axelsson

1. AIM OF THE PRESENT INVESTIGATION

The connective tissue matrix is a proteoglycan gel in which collagen fibrils and scattered cells are embedded. Proteoglycans contain one, two or three different types of glycosaminoglycans covalently bound to a common protein core. There are six types of glycosaminoglycans in connective tissues: chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate, heparan sulfate and hyaluronate. The latter glycosaminoglycan occurs in cartilage and intervertebral discs and is non-covalently associated with proteoglycan monomers.

In the present thesis the connective tissue proteoglycans that contain keratan sulfate are discussed. The aim of the investigation has been to design methods for isolation and characterization of these proteoglycans, to study their chemical composition and physical properties, and to apply the results to the physical properties and biological functions of the tissues.

2. PREVIOUS INVESTIGATIONS

2.1. Early investigations

Most glycosaminoglycans have been purified and characterized during the last forty to fifty years mainly through work by Karl Meyer and coworkers. The term proteoglycan was introduced for the inact macromolecules of glycosaminoglycans and protein. The study of glycosaminoglycans began, however, already in the 19th century. Müller (1836) pointed out the similarities in the chemical composition of cartilage and cornea and described "chondrin", a mixture of organic compounds extracted from cartilage. It was shown that chondrin contained sulfate (Mulder, 1838) and sugar (Fischer & Boedeker, 1861). The polysaccharide later called chondroitin sulfate was first described in the eighties when Krukenberg (1884) extracted an ethanolinsoluble, acidic, organic compound from cartilage and Mörner (1889, 1894) isolated polysaccharides from cartilage ("chondromucoid") and cornea ("corneamucoid").

The presence of glucosamine in corneal polysaccharides was described in 1918 (Levene & López-Suárez) and two decades later a corneal polysaccharide containing mainly equimolar amounts of glucosamine and galactose was described by M. Suzuki (1939). This glycan was isolated and characterized by Meyer et al. (1953) and given the name keratosulfate (from Greek κερας, horn, and Latin sulfur). The name was later changed to keratan sulfate. Polysaccharides with similar (but not identical) composition were detected in human nucleus pulposus (Gardell & Rastgeldi, 1954; Gardell, 1957) and human costal cartilage (Meyer et al.,

1958). Further studies showed that there are two types of keratan sulfate: Corneal keratan sulfate (also called KS I) present in the corneal stroma and skeletal keratan sulfate (KS II) present in cartilage and intervertebral discs.

2.2. Structure of keratan sulfate.

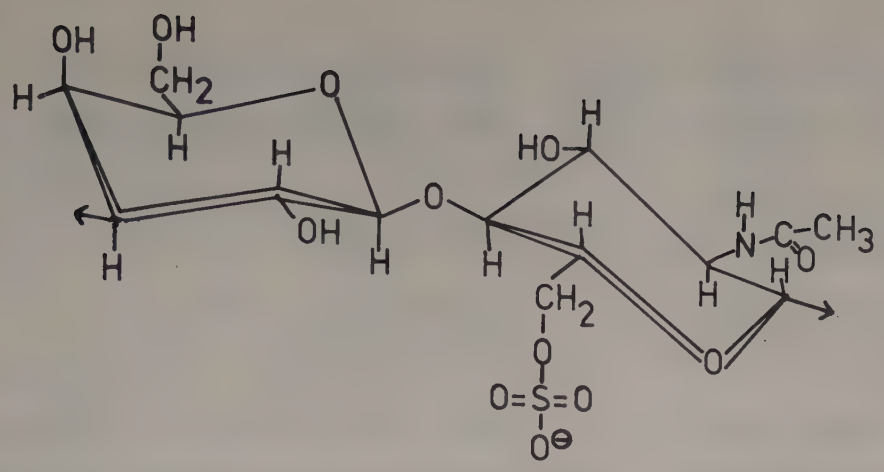
Both corneal and skeletal keratan sulfate contain a repeating unit of (1-3)--β-D-galactopyranosyl-(1-4)-2-acetamido--2-deoxy-β-D-glucopyranose with 6-sulfate groups on most glucosamine residues and some galactose residues (Fig. 1a) (Hirano et al., 1961; Bhavanandan & Meyer, 1967). The structure of keratan sulfate is, however, very complex and our knowledge is fragmentary. Analytical data on keratan sulfate are summarized in Table I and tentative models for the structure are shown in Fig. 1b, c (see also this figure for references). Corneal keratan sulfate chains are considered to be unbranched and linked to the protein core by an alkalistabile N-glycosidic linkage. Papain digestion probably liberates single keratan sulfate chains with a small peptide still attached. Skeletal keratan sulfate chains, on the other hand, are branched with fucose and N-acetylneuraminic acid as non-reducing terminal sugars and linked to the protein core by an alkalilabile O-glycosidic bond. In addition, it has been suggested that there are alkalistabile linkages of unknown structure, possibly involving glutamic acid/glutamine. Papain probably liberates peptides with several attached keratan sulfate chains from skeletal proteoglycans. It is shown in Table I that there are considerable differences between keratan sulfate preparations from different tissues but also between various preparations from the same tissue suggesting heterogeneity and polydispersity.

2.3. Structure of keratan sulfate proteoglycans.

2.3.1. Corneal proteoglycans. The glycosaminoglycan part of corneal proteoglycans from adult mammals and birds contains about 60-65 % glucosaminoglycans and 35-40 % galactosaminoglycans (Anseth, 1961 a; see also Table II). Low-sulfated fractions of chondroitin sulfate have been isolated and referred to as chondroitin (Davidson & Meyer, 1954). Heparan sulfate and hyaluronate have not been detected in adult mammalian corneal stroma but heparan sulfate is synthesized in embryonic cornea (Hart, 1976) and by cultured stroma and endothelial cells (Yue et al., 1976) while hyaluronate is the predominant glycosaminoglycan in cultures of corneal stroma cells (Dahl et al., 1974; Gnädinger & Schwager-Hübner, 1975a, b; Yue et al., 1976; see also Fig. 2). The ratio of chondroitin 6-sulfate to chondroitin 4-sulfate is much higher in the early embryonic chick cornea than later in the development (Hart, 1976).

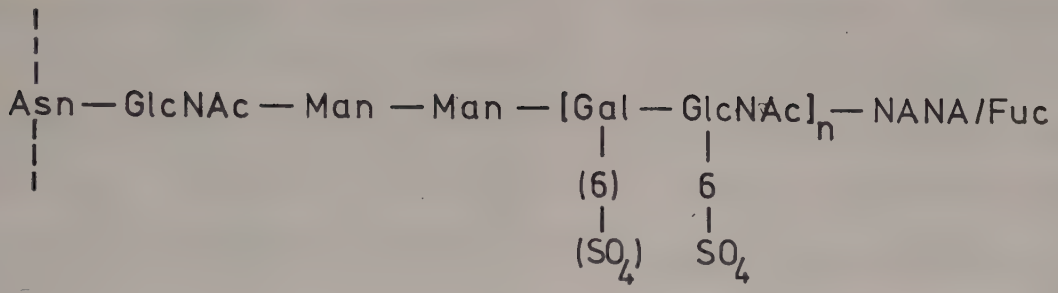
All previously described proteoglycan fractions isolated from adult cornea contain both keratan sulfate and galactosaminoglycans (Stuhlsatz et al., 1971; Muthiah et al., 1974; Bettelheim & Plessy, 1975). Corneal proteoglycans are considerably smaller than cartilage proteoglycans; the molecular weights are of the order 10^5 (Bettelheim & Plessy, 1975). The average protein and keratan sulfate contents are higher in corneal proteoglycans than in cartilage proteoglycans (Stuhlsatz et al., 1971; Muthiah

(a)



β,1←3 D-Galactose β,1–4 GlcNAc-6-SO_4 β,1→3

(b)



(c)

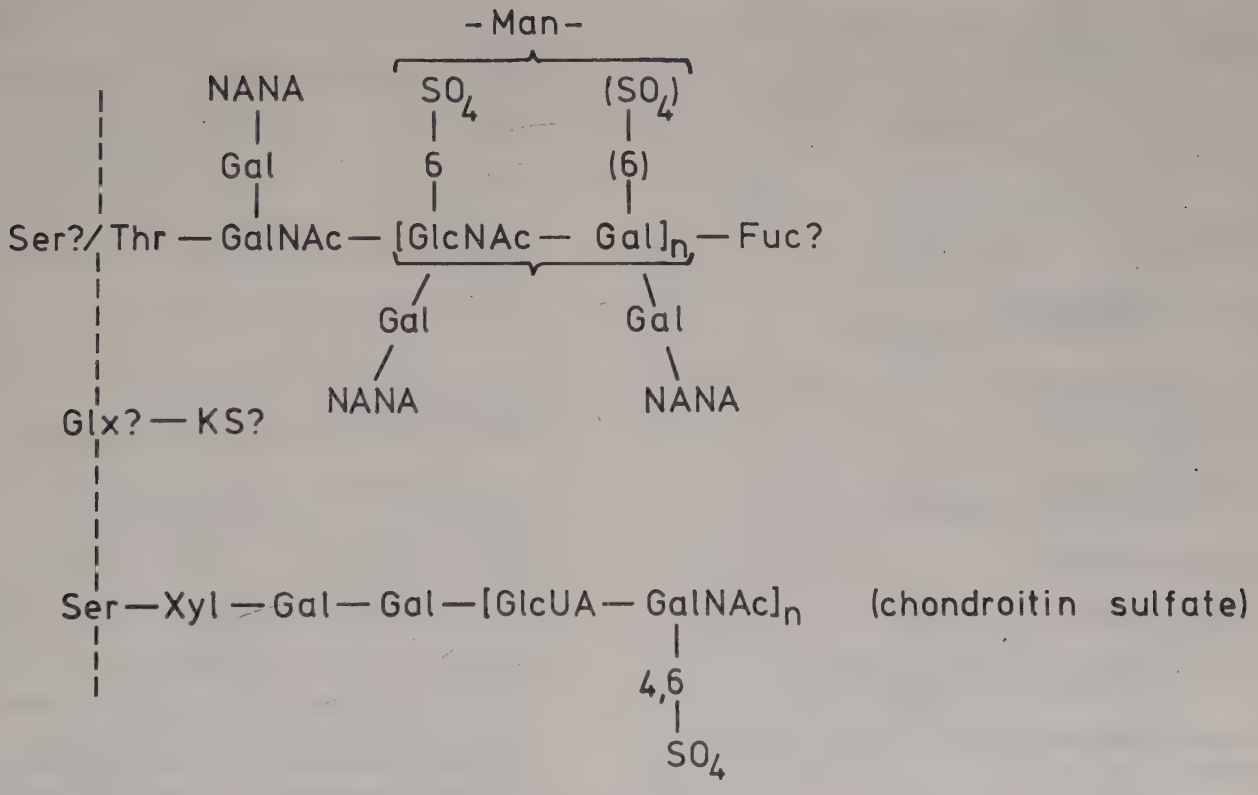


Fig. 1. Structure of keratan sulfate.

(a) The disaccharide unit of keratan sulfate. (b) Hypothetical structure of corneal keratan sulfate according to Stuhlsatz et al. (1971) and Baker et al. (1975). (c) Hypothetical structure of skeletal keratan sulfate according to data from Bray et al. (1967), Rodén (1968), Greiling et al. (1970), Heinegård (1972b) and Hopwood & Robinson (1974a, b)

TABLE I. DATA ON KERATAN SULPHATE.

Sources and references	Molecular weight[a]	Partial specific volume (ml/g)	GalNAc / GlcNAc	Gal / GlcNAc	Sulfate / $HexNH_2$	Mannose	Fucose	NANA
			Molar ratios			% (w/w) or residues per glycan chain (r/c)		
BOVINE CORNEAL KERATAN SULPHATE								
Anseth & Laurent (1961); Laurent & Anseth (1961)	4000-19000 (M_w)	0.47-0.55		1.1-1.7[b]	0.88-1.04			
Seno et al. (1965)			1:50		1.1		0.7-1.0%	0.2-1.0%
Mathews & Cifonelli (1965)				0.84	1.08-1.22		<0.7%	
Greiling & Stuhlsatz (1966)	8600-19800 (M_w)	0.5	1:160	0.98-1.01	0.96-1.19		1.2-1.6%	0.40-0.67%
Fransson & Anseth (1967)				1.02-1.23[c]	0.87-2.0			
Saliternik-Givant & Berman (1970)			1:7	0.94-1.67	0.85-1.70			1.6-3.0%
Stuhlsatz et al. (1971)					0.9 -1.2	2.4-3.6% (2 r/c)		
Plessy & Bettelheim (1975); Bettelheim & Plessy (1975)	7300 (M_w)	0.63			1.09			
Choi & Meyer (1975)	11900 (M_n)			1.05		2 r/c		
Baker et al. (1975)				0.89	1.07	3.1%	0.5%	
SKELETAL KERATAN SULPHATE								
Old human rib cartilage								
Seno et al. (1965)			1:11		1.3-1.5		1.5-3.7%	0.3-3.0%
Choi & Meyer (1975)	11000 (M_n)			1.21		1.5 r/c		
Bovine nasal cartilage								
Hascall & Riolo (1972)	4000 (M_w)	0.60	1:6.6	1.03		0.5 r/c		
Robinson & Hopwood (1973); Hopwood & Robinson (1973)	7500 (M_n); 3000-22000 (M_n)							
Human intervertebral disc								
Pearce & Grimmer (1976)	6000-18000 (Mg)							
Human nucleus pulposus								
Antonopoulos et al. (1969)			1:4-1:49	1.06-1.26	1.03-1.21			
Pig nucleus pulposus								
Choi & Meyer (1975)	11900 (M_n)			1.29		1 r/c		
Bovine intervertebral disc								
Robinson & Hopwood (1973); Hopwood & Robinson (1973, 1974a,b)	18600 (M_w); 7000-47000 (M_n)					1 r/c	1 r/c	3 r/c
Rabbit femur bone tissue								
Masubuchi et al. (1975)				1.17-1.18	0.40-0.52		2.2-2.4%	6.0-7.6%

[a] M_w, weight average molecular weight, M_n, number average molecular weight; Mg, approximative molecular weight from gel chromatography

[b] Ratio of hexose to hexosamine

[c] Ratio of neutral sugar to glucosamine.

et al., 1974; Bettelheim & Plessy, 1975).

2.3.2. Skeletal proteoglycans. Cartilage and intervertebral disc proteoglycans have been more extensively studied than corneal proteoglycans. The present knowledge on the structure and properties of cartilage proteoglycans is partly summerized in paper V. All proteoglycans sofar isolated with mild methods from cartilage and intervertebral disc contain both chondroitin sulfate and keratan sulfate.

Proteoglycans from bovine nasal cartilage have been used in a large number of studies on the structure of proteoglycans and their structure is better established than that of proteoglycans from any other source. The monomers have an average composition of about 87 % chondroitin sulfate (mostly chondroitin 4-sulfate), 6 % keratan sulfate, and 7 % protein, and their molecular weights are in the range 1-4 x 10^6 (Hascall & Sajdera, 1970). The glycosaminoglycan chains are bound to one region of the protein core (about 60 % by weight of the protein core) while the rest of the protein core forms a structure which can interact noncovalently with hyaluronate. Large aggregates containing several proteoglycan monomers bound to one hyaluronate molecule can therefore be formed (Hardingham & Muir, 1972; Hascall & Heinegård, 1974a, b; Heinegård & Hascall, 1974). Some experimental data indicate that proteoglycan monomers contain keratan sulfate-enriched regions (Heinegård, 1972b).

There is good evidence that proteoglycans from the intervertebral disc have the same general structure as proteoglycans from cartilage although they are smaller, have a higher keratan sulfate content and contain a large fraction of proteoglycan molecules that do not bind to hyaluronate (Emes & Pearce, 1975; Axelsson et al., 1976; Hardingham & Adams, 1976).

Very little is known about the small amounts of proteoglycans in bone tissue and they have not been studied in the present investigation.

2.4. Keratan sulfate in tissues

2.4.1. Cornea. The vertebrate cornea is built up by five layers: an external epithelium with several layers of cells, Bowman's membrane, a stroma (which comprises more than 90 % of the thickness of the cornea), Descemet's membrane and a single layer of endothelial cells. The two membranes are mainly collagen; Descemet's membrane is composed of a basement membrane type of collagen synthesized by the endothelial cells (Kefalides et al., 1976). In mammals, the stroma contains about 78 % water, 15 % collagen, 5 % other proteins, 1 % glycosaminoglycans and 1 % salts (Maurice, 1969; Mathews, 1975). It is a specialized type of connective tissue built by lamellae of parallell collagen fibrils and proteoglycans. Between the lamellae are modified fibroblasts called keratocytes or stroma cells which occupy 5-10 % of the volume of the tissue. Corneal collagen has the structure $\left[\alpha 1(I)\right]_2 \alpha 2$ (Trelstad, 1974).

The normal corneal stroma is underhydrated due to the endothelial cells which actively pump water from the stroma to the aqueous humor (for review, see Maurice, 1969). Keratan sulfate proteoglycans bind larger amounts of water than chondroitin sulfate proteoglycans and loose the water more easily than the chondroitin sulfate proteoglycans (Bettelheim & Plessy, 1975; Plessy & Bettelheim, 1975). Studies

on the topographical distribution of glucosamine and galactosamine suggest that keratan sulfate proteoglycans are concentrated to the posterior part of the stroma, close to the endothelial cells, while the chondroitin sulfate proteoglycans are concentrated to the anterior parts of the stroma (Bettelheim & Goetz, 1976). Keratan sulfate proteoglycans may facilitate the transport of water in the stroma while chondroitin sulfate proteoglycans store a limited amount of tightly bound water and accordingly prevent water loss from the epithelial surface of cornea (Bettelheim & Goetz, 1970).

Transparency is the most important property of cornea. Maintenance of a carefully regulated underhydration and a quasi-regular, quasi-random system of thin collagen fibrils of uniform diameter is probably vital for corneal transparency (Maurice, 1957; Langham et al., 1969). The diameter of normal fibrils is much smaller than the wavelength of visible light; therefore, they cause negligible scattering (Benedek, 1971). Aggregation of several collagen fibrils to a coarse fibril or appearance of "lakes" free from fibrils causes light scattering and opacity (Langham et al., 1969; Schwarz & Graf Keyserlingk, 1969; Benedek, 1971; Borcherding et al., 1975). Several authors have suggested an important role for proteoglycans in developement and maintenance of corneal transparency. The appearance of corneal transparency during ontogeny is coincident with a considerable increase in high molecular weight and/or highly sulfated keratan sulfate (Fig. 2; Anseth, 1961b: Anseth & Fransson, 1970; Hart, 1976).

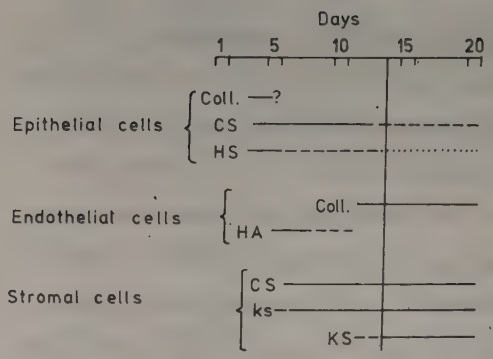


Fig. 2. Prenatal development of chick cornea.

The periods the cells synthesize the various macromolecules are shown. For keratan sulfate, low molecular weight and/or low sulfated fractions (ks) and high molecular weight and/or highly sulfated fractions (KS) are shown. The vertical line indicates the appearance of transparency, underhydration and metachromatic staining of cornea. Data were compiled from Anseth (1961b), Meier & Hay (1973, 1974), O´Rahilly (1975) and Hart (1976).

Corneal edema due to endothelial or epithelial injury or various diseases is accompanied by corneal clouding and a concomitant decrease in the concentration of chondroitin sulfate and keratan sulfate (measured as % w/w of dry tissue). Persistant opacity is associated with persistent low levels of glycosaminoglycans; restitution of transparency is associated with restitution of normal concentrations of chondroitin sulfate and keratan sulfate (Anseth & Fransson, 1970). Most investigators presume that the proteoglycans prevent aggregation of the collagen fibrils (Langham et al., 1969; Hodson, 1971; Kaye et al., 1976).

TABLE II. OCCURRENCE AND CONCENTRATIONS OF GLYCOSAMINOGLYCANS IN ADULT MAMMALIAN TISSUES THAT CONTAIN KERATAN SULFATE.

Some values are calculated from data on hexosamine concentration assuming a hexosamine concentration of 40 % (w/w) in hyaluronate and 25 % (w/w) in other glycans.

Tissue and references	Keratan sulfate	Chondroitin sulfate	Dermatan sulfate	Hyaluronate
	Concentration (% w/w, dry tissue)			
Bovine cornea (Anseth & Laurent, 1961; Stuhlsatz et al., 1972; Handley & Phelps, 1972; Hart, 1976)	3.6	1.6[a]	0.5	
Horse nasal cartilage (Antonopoulos et al., 1964	8	40		1
Human nucleus pulposus (Antonopoulos, 1965)	23	13[b]		4
Rabbit femoral bone tissue (Masubuchi et al., 1975)	0.3	0.3[a]		

[a] Mainly chondroitin 4-sulfate [b] Mainly chondroitin 6-sulfate

2.4.2. Cartilage. Cartilage is a mesenchymal tissue composed of a proteoglycan gel which contains a three dimensional network of randomly distributed, thin collagen fibrils and scattered cells. The collagen is of type II in hyaline cartilage and type I in fibrocartilage. The cells are called chondrocytes. They occupy 1-10 % of the tissue volume (Mathews, 1975). Wet cartilage contains 10-20 % (w/w) collagen and 4-8 % (w/w) proteoglycans (Mathews, 1975). The proteoglycans of hyaline cartilage contain chondroitin 4-sulfate and keratan sulfate and are non-covalently linked to hyaluronate (Fig. 1c; Table II; paragraph 2.3.2). Proteoglycans from the fibrocartilage of human knee joint meniscus contain chondroitin sulfate and dermatan sulfate (Habuchi et al., 1973).

Proteoglycans from bovine nasal cartilage occupy a domain of 55 ml/g in aqueous solutions (Gerber & Schubert, 1964; Hascall & Sajdera, 1970) which means that the domains overlap when the proteoglycan concentration exceeds 2 %. Since the proteoglycan concentration in cartilage is higher (up to 10 %), cartilage proteoglycans are underhydrated *in vivo*. The elasticity of cartilage is to a large extent dependent on the underhydration and mutual steric exclusion and electrostatic repulsion of proteoglycans (Mathews, 1975; Scott, 1975).

2.4.3. Intervertebral disc. The intervertebral discs form symphysis (i.e. joints mostly consisting of fibrocartilage) that connect the vertebral bodies. It is shown in Table II that nucleus pulposus (the central, gelatinous part of the disc) has the highest keratan sulfate concentration of all tissue in the body. Anulus fibrosus (the peripheral, fibrous part of the disc)

has a lower total content of glycosaminoglycans (Antonopoulos et al., 1964; Adams & Muir, 1976). There are considerable differences in glycosaminoglycan composition between discs from different levels of the vertebral column, from different ages and from different species (Antonopoulos et al., 1964; Antonopoulos, 1965; Adams & Muir, 1976; Hardingham & Adams, 1976). Type II collagen (typical for hyaline cartilage) is the predominant type of collagen in nucleus pulposus while type I (typical for fibrocartilage) is predominant in anulus fibrosus (Eyre & Muir, 1976).

Nucleus pulposus is the shock absorber of the vertebral column. Its elastic resistance to compressive forces is due to "inflation" of the tissue matrix by the proteoglycans as described for cartilage in the preceding paragraph.

2.4.4. Bone. When bone is formed by mineralization of the epiphyseal plates, there is a sharp decrease in the concentration of keratan sulfate and chondroitin 6-sulfate and a less pronounced decrease in the concentration of chondroitin 4-sulfate (Lohmander & Hjerpe, 1975). Thus, chondroitin 4-sulfate would be the predominant glycosaminoglycan in bone. According to one report (Masubuchi et al., 1975) there are equal amounts of keratan sulfate and chondroitin sulfate in bone (Table II). There are evidences that proteoglycan aggregates (but not proteoglycan monomers) inhibit the growth of bone mineral nuclei in the epiphyseal plate and thus play a role in the regulation of bone growth and calcification (Howell & Pita, 1976).

2.4.5. Other tissues. According to preliminary, unconfirmed reports there may be keratan sulfate or keratan sulfate-like glycans in pig aorta (Franek & Dunstone, 1968), human aorta (Stuhlsatz et al., 1976) and rat brain (Vitello et al., 1976).

3. PRESENT INVESTIGATION.

3.1. Methods for isolation and characterization of proteoglycans.

3.1.1. Extraction. The extraction procedure throughout the present investigation has been stirring of tissue pieces with 4 M guanidinium chloride - 0.05 M sodium acetate buffer (pH 5.8). Sajdera and Hascall (1969) showed that such extraction procedures give high yields (about 85 %) of proteoglycans. It is shown in paper I that the same high yields are obtained when proteoglycans are extracted from cornea, sclera and aorta with this method. The yield is only 45 % when corneal proteoglycans are extracted with high speed homogenization of the tissue in water (Stuhlsatz et al., 1971). Intervertebral disc proteoglycans (paper VI) have also been extracted using the procedure described by Sajdera & Hascall; the yield of proteoglycans was over 85 % (Axelsson et al., 1976; Inerot & Heinegård, personal communication). Earlier used procedures for extraction (high speed homogenization in water or dilute salt solutions) resulted in degradation of proteoglycans by tissue enzymes and/or mechanical forces (Sajdera & Hascall, 1969). Such degradation can be minimized if tissue pieces are gently agitated in 4 M guanidinium chloride. Oegema et al. (1975), however, found that even when this method was used the proteoglycans extracted from Swarm rat chondrosarcoma were degraded. Since the degradation could be inhibited by addition of protease inhibitors (EDTA, 6-aminohexanoic acid and benzamidine) to the guanidinium chloride solution, it was concluded that proteases produced by the tumor were causing the degradation. In control experiments with normal cartilage and intervertebral discs no differences could be discovered between proteoglycans extracted with solutions containing protease inhibitors and with guanidinium solution alone provided all procedures are performed in the cold (D. Heinegård et al., unpublished)

3.1.2. Purification. Density gradient centrifugation of tissue extracts (Franek & Dunstone, 1966; Sajdera & Hascall, 1969; Heinegård, 1972a) is the method of choice for purification and fractionation of proteoglycans from cartilage and intervertebral disc and it was therefore used in the present investigation (I, V, VI). Proteoglycans from other tissues with lower concentration of proteoglycans are more conveniently purified by ion exchange chromatography. In such procedures the volume of the extract is not critical and glycoproteins are better separated from protein-rich proteoglycans which have low buoyant densities. A procedure was designed where the good extraction yield obtained with guanidinium chloride solutions was utilized. It was necessary to exchange the guanidinium chloride for urea before the ion exchange chromatography. The chromatography was performed at $+4^{\circ}C$ since at this low temperature cyanate formation is drasticly reduced (Hagel et al., 1971). The urea solutions were buffered with an amino buffer (Tris) which neutralizes both cyanate and hydroxyl ions (Hirs, 1967). It is shown in paper I that this preparation method is a simple and efficient method for purification of proteoglycans from several types of connective tissue. The presence of urea is essential for the recovery of sample since in urea denaturated proteins like collagen are retained in solution. Bettelheim and Plessy (1975) used chromatography on DEAE-Sephadex in the absence of urea for purification of corneal proteoglycans. Their

yield of proteoglycans was only 16 % of that described in paper I probably because of coprecipitation of proteoglycans with collagen during dialysis of the extract.

3.1.3. Gel chromatography. Gel chromatography was the principal method used for fractionation and characterization of protoeglycans in the present investigation. It was thus used both for preparative and analytical purposes while ion exchange chromatography is less useful for fractionation and characterization of proteoglycans because of their high charge density. Some of the factors of importance for interpretation of data from gel chromatography are discussed in a separate paper (II). Chromatography of various proteins indicated that asymmetric molecules are more retarded than expected from the theoris for gel chromatography (for a discussion of the theory for gel chromatography see Laurent & Killander, 1964). Similar results were recently discussed by Nozaki et al. (1976). The volume of agarose fibers calculated from calibration of agarose gels with globular proteins is very large (II, Table III). Such data are inconsistant with the fact that small molecules are excluded from only a few per cent of the gel volume. An explanation for the conflicting observations is that agarose fibers are branched while the theory assumes unbranched fibers. In conclusion, it is necessary to consider the effects of molecular asymmetry and exclusion of large molecules from fiber branching points when data from gel chromatography are interpreted.

The experimental results described in paper II and results from investigations of gel chromatography in denaturating solvents (see paper II for references) were applied to corneal proteoglycans which were chromatographed on Sepharose 4B columns in various associative and dissociative buffers (IV) and to keratan sulfate peptides from skeletal proteoglycans (VI).

3.1.4. Ultracentrifugation. Ultracentrifugation techniques were also used for physical characterization of proteoglycans. Molecular weight determinations were made by sedimentation equilibrium centrifugation (Yphantis, 1964; Chervenka, 1970) in a Beckman-Spinco model E centrifuge using interference optics. Linear plots of ln fringe displacement versus the square of radial distance were obtained for both corneal keratan sulfate proteoglycans in 6 M guanidinium chloride (IV) and keratan sulfate peptides from cartilage proteoglycans in 0.25 M NaCl (V) at several concentrations and rotor speeds. Such linear plots are often considered as proof for monodispersity of the sample. In these experiments, a considerable polydispersity was revealed by gel chromatography. In addition, a lower average molecular weight for corneal proteoglycans was obtained at a higher rotor speed. Yphantis (1964) pointed out that non-ideality under unfavourable circumstances may obscure polydispersity. This must be considered in the interpretation of data from sedimentation equilibrium centrifugation of proteoglycans.

Further information on the polydispersity of corneal proteoglycans was obtained by sedimentation velocity centrifugation in various solvents. Beckman-Spinco model E centrigufes equipped with conventional schlieren optics were used initially. The latest experiments were made with an MSE Centriscan 75 centrifuge using the schlie-

ren scanner.

The corneal proteoglycans were also characterized by transport centrifugation as described by Pita & Müller (1973) using a Sorvall OTD-2 centrifuge. This centrifuge has the necessary properties for this technique: a precise control of rotor speed and a smooth, symmetric acceleration and retardation. In conventional analytical ultracentrifugation, the sedimentation and diffusion of samples are followed by measurements of refractive index or ultraviolet light absorption and no information on the chemical nature of the sample is obtained. In the Pita-Müller technique, on the other hand, several analytical methods (e.g. determinations of protein concentration, neutral sugar concentration, radioactivity, enzymic activity, etc.) may be used. In addition, the analytical methods may allow very low sample concentrations giving negligible aberrations of physical-chemical properties from the ideal behavior of infinitely dilute solutions (Müller, Pita & Hascall, personal communication). In the present investigation, the distribution of protein in the centrifuge tubes were measured after centrifugation of corneal proteoglycans at pH 4 and pH 7 (IV).

3.2. Bovine corneal proteoglycans.

3.2.1. Distribution of keratan sulfate in corneal proteoglycans. The preparation of corneal proteoglycans obtained by guanidinium chloride extraction and DEAE-cellulose/urea chromatography was fractionated by various methods in order to study the heterogeneity and polydispersity of the proteoglycans. Gel chromatography (paper III, Fig. 4a), density gradient centrifugation (III, Fig. 3), and ion exchange chromatography (III, p. 495) gave fractions with significant but small differences in the ratios between keratan sulfate, galactosaminoglycans, oligosaccharides and protein. Fractionation of proteoglycans by ethanol precipitation, however, gave fractions with very different composition (III, Tables 1-3) and was therefore used in an initial fractionation; subfractionation was performed by gel chromatography (III, Fig. 4 b-d; IV). About 25 % of the isolated proteoglycans were recovered in a fraction (70P) which contained keratan sulfate as the only glycosaminoglycan (III). No other example of a pure keratan sulfate proteoglycan has been described previously. Fraction 70P contained 45-55 % of the keratan sulfate in the tissue. Another fraction (50P) contained about 33 % of the isolated proteoglycans but only 5-10 % of the keratan sulfate present in cornea (III). It was possible to prepare subfractions of 50P which had a galactosaminoglycan to glucosaminoglycan ratio of 13:1 (III, Fig. 4, legend) and thus were very much enriched in galactosaminoglycans. The third fraction (60P) was intermediate to 50P and 70P with respect to glycosaminoglycan and amino acid composition (III).

The galactosaminoglycan-containing fractions 50P and 60P were characterized by analysis of amino acid, neutral sugar and uronic acid composition (III), gel chromatography of proteoglycans and glycan-peptides liberated by papain (III), dissociative density gradient centrifugation and dissociative gel chromatography before and after chondroitinase digestion (Axelsson & Heinegård, 1976), and analytical ultracentrifugation (unpublished). The results indicate that most of the oligosaccharides in 50P and 60P and some of the keratan sul-

fate chains in 50P are covalently bound to the same protein core to which also galactosaminoglycan chains are covalently bound. Most of the keratan sulfate chains in 50P and 60P, however, are constituents of proteoglycans which contain no detectable amounts of galactosaminoglycans (Axelsson & Heinegård, 1976). The average molecular size of the proteoglycans in 50P and 60P is larger than in 70P (III, Fig. 4). Surprisingly, however, keratan sulfate is enriched in the largest molecular size fractions of 50P (III, Fig. 4b, legend). There were no differences in the gel chromatographic pattern of the keratan sulfate peptides obtained by papain digestion of 60P and 70P (III, Fig. 5).

3.2.2. Characterization of corneal keratan sulfate proteoglycans. Fraction 70P, which contains keratan sulfate but no other glycosaminoglycans, was characterized by gel chromatography, gel electrophoresis and analytical ultracentrifugation in various solvents (IV). When the proteoglycans were dissolved in physiological saline, they could be separated in two polydisperse populations with similar chemical composition but different average molecular size and molecular weight (III, IV).In dissociative solvents like 4-6 M guanidinium chloride or 1 % sodium dodecyl sulfate solutions the major portion of the large molecular size fraction (70PA) could be dissociated to monomers with virtually the same molecular size as the small molecular size fraction (70PB) (IV). A large proportion of the proteoglycans in fraction 70PB was associated to aggregates with the same molecular size as 70PA when pH was lowered or after reduction and alkylation; this aggregation required the presence of 70PA (IV). Very approximative average values for the physical parameters of 70P in concentrated guanidinium chloride solutions were: Molecular weight, 72000; Stokes' radius, 12 nm; sedimentation coefficient at zero concentration ($s^{o}_{20,w}$), 1.7 S; frictional ratio, 4.5 (IV). There are some independent experiments which suggest that the three-dimensional structure of the protein core is partly determined by intra-chain S-S bridges (IV, Figs. 5, 8, Table 1). Most proteoglycan molecules contain one, two or three keratan sulfate chains and several closely associated, possibly covalently linked oligosaccharide chains which contain the same sugars as the linkage region of corneal keratan sulfate and chondroitin sulfate (IV). Isolated keratan sulfate linkage regions contained near equimolar amounts of glutamic acid/glutamine and aspartic acid/asparagine but very little of leucine (IV, Table 3), the predominant amino acid of 70P (III, Table 2), or other amino acids with hydrophobic side chains.

The conformation and the degree of aggregation of the proteoglycans in vivo are not known. Mild methods for isolation of proteoglycans have been used throughout the present investigation; nevertheless, one must always consider the possibilities for degradation and modification of proteoglycans in vitro. It is shown in paper IV that the aggregation of 70PB molecules is facilitated by conditions which cause denaturation of proteins (low pH; reduction; treatment with sodium dodecyl sulfate). This may suggest that 70PA at neutral pH partly represents denaturated proteoglycans.

It is shown in an investigation of corneal proteoglycans (Muthiah et al., 1974) published after submission of paper III that keratan sulfate-rich proteoglycans have higher average buoyant densities

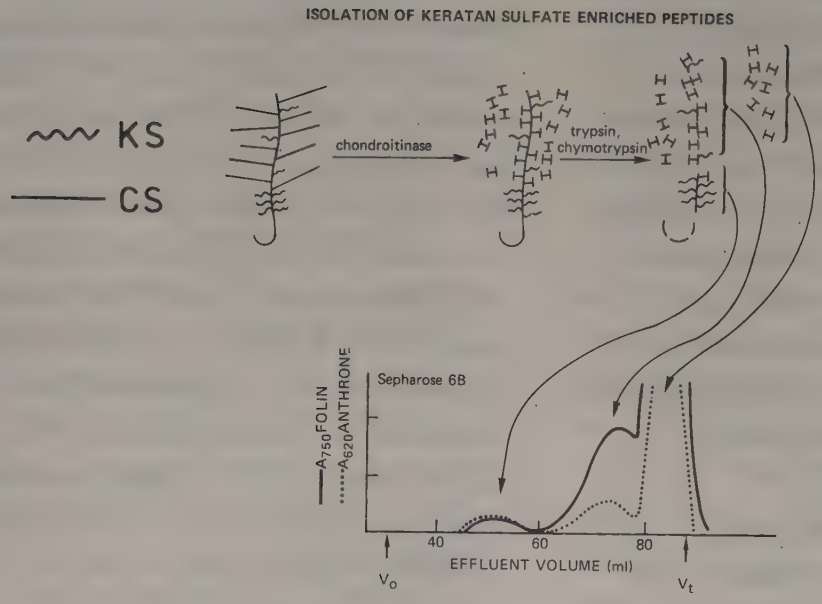
in CsCl solutions and higher average K_d on Sepharose 4B than chondroitin sulfate-rich proteoglycans and that the material completely excluded from Sepharose 4B mostly consists of protein; these findings are confirmed in paper III. However, Muthiah and coworkers did not succeed to separate keratan sulfate proteoglycans, galactosaminoglycan-rich proteoglycans and glycogen. Consequently, the interpretation of their data is difficult.

3.3. Bovine skeletal proteoglycans.

3.3.1. Distribution of keratan sulfate in skeletal proteoglycans. It has been shown that glycosaminoglycan chains are located to certain regions of the protein core of the proteoglycans (see paragraph 2.3.2.). It has, however, not been shown if there are differences in the distribution of keratan sulfate and chondroitin sulfate in the proteoglycan. The distribution of keratan sulfate was therefore studied by fractionation of the peptides obtained after enzymic and chemical degradation of proteoglycans from bovine nasal and tracheal cartilage (V, VI) and bovine intervertebral disc (VI). The combined action of chondroitinase ABC, trypsin and chymotrypsin yields three main populations of degradation products which can be separated by agarose gel chromatography (Fig. 3a). The first peak contains peptides with several keratan sulfate chains and few or no chondroitin sulfate linkage regions. The second peak contains mainly peptides with several chondroitin sulfate linkage regions and few or no keratan sulfate chains. The third peak contains the chondroitin sulfate disaccharides derived from chondroitin sulfate by the action of chondroitinase and also small peptides. The results indicate that the proteoglycans contain keratan sulfate-rich regions with little chondroitin sulfate and chondroitin sulfate-rich regions with little keratan sulfate. The localization of these regions was studied by degradation of proteoglycan aggregates with trypsin and hydroxylamine. The part of the proteoglycan which remained attached to hyaluronate after degradation was further studied. After trypsin digestion this fragment consisted primarily of protein with small amounts of keratan sulfate (paper V, Fig. 12). After hydroxylamine treatment, the hyaluronate-bound fragment contained a large portion of the keratan sulfate and protein from the native proteoglycan but little chondroitin sulfate (paper V, Fig. 11, Tables IV, V). The experiments discussed in paper V suggest that there are at least three regions of the proteoglycan: firstly, a hyaluronate-binding protein region with little keratan sulfate and no detectable chondroitin sulfate (Heinegård & Hascall, 1974); secondly, an adjacent keratan sulfate-rich region which contains approximately 60 % of the total amount of keratan sulfate present in the native proteoglycan but only about 10 % of the chondroitin sulfate; and thirdly, a chondroitinsulfate-rich region which contains about 90 % of the chondroitin sulfate chains but only about 20 % of the keratan sulfate chains (V). These data have been summarized in tentative models for the structure of proteoglycan monomers (paper V, Fig. 14;) and proteoglycan aggregates (Fig. 3b).

3.3.2. Characterization of the keratan sulfate-rich region of skeletal proteoglycans. The weight average molecular

(a)



(b)

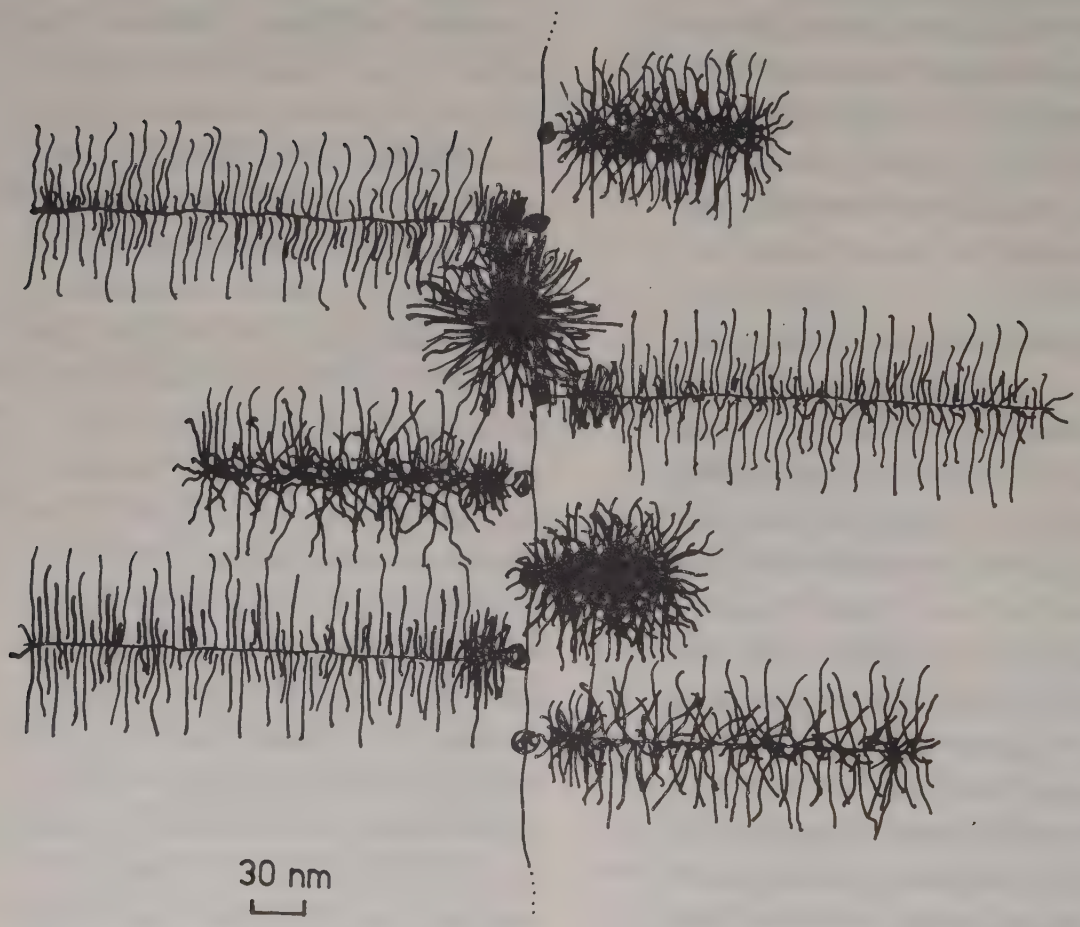


Fig. 3. Models for the structure of native and degraded cartilage proteoglycans. (a) Stepwise degradation of proteoglycan monomers and separation of the peptides by gel chromatography. Only a small number of the glycosaminoglycan chains of the molecule is shown in the figure. (b) Hypothetical model showing a part of a proteoglycan aggregate with proteoglycan monomers bound to a central hyaluronate molecule.

weight of the keratan sulfate peptides obtained by digestion of bovine nasal cartilage proteoglycans with chondroitinase ABC, trypsin and chymotrypsin is 122 000 (V, Fig. 4). The intact proteoglycan monomers contain about 6 % keratan sulfate which contains about 25 % glucosamine and the average molecular weight of the preotoglycan monomers is 2.5×10^6 (Hascall et al., 1972). Therefore, the total mass of all the keratan sulfate in one proteoglycan molecule is about 150 000 daltons. About 60 % of the keratan sulfate is located in the keratan sulfate-rich region (V) corresponding to a total mass of about 90 000 daltons. The glucosamine concentration in the keratan sulfate peptides is 15.3 % (calculated from Table I in paper V) which would correspond to a keratan sulfate content of about 61 %, i.e. a keratan sulfate mass of 74 000 daltons per keratan sulfate peptides. The true mass is probably slightly higher since the analytical data are not corrected for salt or water contents. Thus, the keratan sulfate-rich regions and the keratan sulfate peptides are of about the same size.

Intervertebral disc proteoglycans have the same general structure as cartilage proteoglycans (V; Axelsson et al., 1976). The keratan sulfate peptides isolated after chondroitinase ABC, trypsin and chymotrypsin digestion of proteoglycans from bovine nucleus pulposus are larger than corresponding fragments from nasal cartilage proteoglycans (VI). It is possible that the keratan sulfate regions are larger in proteoglycans from nucleus pulposus compared to cartilage proteoglycans. There are also evidences from gel chromatography and end-group analysis that the individual keratan sulfate chains isolated from keratan sulfate-rich peptides after alkali treatment are larger in intervertebral disc proteoglycans compared to nasal cartilage proteoglycans (VI) corroborating data in the literature (Table I).

3.4. Physiological implications of the present investigation.

It is shown in the present investigation that cornea contains a large population of proteoglycans with keratan sulfate as the only glycosaminoglycan (III). It is also shown that these proteoglycans are smaller and show a more narrow size distribution than the other proteoglycans. It is therefore possible that the keratan sulfate proteoglycans have a different turnover rate, different biological functions and higher diffusion rates in the corneal stroma than other proteoglycans. Maurice (1957) studied the diffusion of globular proteins through the bovine corneal stroma which he considered to contain a collagen fibril network with 31 nm fibril diameter and 31 nm interfibrillar gaps. He found that "the diameter of the largest molecules which can diffuse in the stroma in its normal state is, then, probably of the order of 12 mμ." A molecular radius above 6 nm would consequently be associated with a very low diffusion constant in the corneal stroma. The keratan sulfate proteoglycan monomers (70PB) have an average Stokes' radius of about 12 nm (in concentrated guanidinium chloride) but the radius of the "equivalent sphere" in gel chromatography is about 7 nm (IV). It is likely that the latter radius is more relevant to the diffusion

rate in the corneal stroma (II). It may be concluded that the average size of the keratan sulfate proteoglycan monomers is near the critical size to allow diffusion in the stroma. Aggregation of the monomers would probably result in immobilization of the aggregates while degradation of the monomers immediately would yield fragments which would easily be removed from the corneal stroma by diffusion.

The proteoglycans "inflate" the collagen fibril meshwork of cartilage and intervertebral discs and hence provide resistance to compressive forces (paragraph 2.4.2). This inflation is due to mutual electrostatic repulsion and steric exclusion and is thus influenced by the distribution of the polyanionic glycosaminoglycan chains in the proteoglycans. The different properties of the three regions of skeletal proteoglycan molecules must also be considered when the interactions between proteoglycans and mineral phase nuclei in calcifying tissue (paragraph 2.4.4) or the interactions between proteoglycans and collagen are studied. The keratan sulfate-rich region is relatively resistant to some proteases (paragraph 3.3.2). The various regions of the skeletal proteoglycans may also have different susceptibility for tissue proteases.

4. SUMMARY

1) Proteoglycans were extracted with 4 M guanidinium chloride (pH 5.8) from bovine corneal stroma, nasal septum cartilage, tracheal cartilage, intervertebral disc, sclera, and aorta. More than 85 % of the glycosaminoglycans in the tissues were extracted. A procedure was worked out for the purification of proteoglycans from various tissues by using ion exchange chromatography in the presence of 7 M urea. The chemical composition and molecular size of proteoglycans from the different tissues were compared. It was found that proteoglycans from cartilage were the largest and had the lowest protein contents. Sclera and aorta contained proteoglycans of intermediate sizes and protein contents, while cornea contained proteoglycans with high protein contents and small sizes. Further studies were made with keratan sulfate-containing proteoglycans from cornea, cartilage, and intervertebral disc.

2) Gel chromatography and analytical ultracentrifugation were the principle methods used for characterization of the proteoglycans. It was concluded from model experiments with gel chromatography of well-characterized proteins that the partition coefficients (K_{av} and K_d) used when macromolecules are characterized by gel chromatography are not exclusively determined by Stokes' radius of the molecules but also by the shape of the molecules and the branching of the gel fibers.

3) Proteoglycans from cornea were further fractionated by ethanol precipitation and gel chromatography. Half of the corneal keratan sulfate could be recovered in a proteoglycan fraction free from other glycosaminoglycans. A smaller subfraction of

the proteoglycans contained a more than tenfold excess of galactosaminoglycans (chondroitin sulfate and dermatan sulfate) over keratan sulfate. The keratan sulfate proteoglycan fraction was characterized by sedimentation equilibrium centrifugation and sedimentation velocity centrifugation in 6 M guanidinium chloride. The weight average molecular weight was 72 000 and Stokes' radius about 12 nm. The material formed larger aggregates under certain conditions like low pH. It contained oligosaccharides with the same carbohydrate constituents as corneal glycosaminoglycan-protein linkage regions. It was demonstrated that the protein core contained disulfide bridges.

4) Proteoglycans from cartilage and intervertebral disc were purified by density gradient centrifugation. By using various fragmentation techniques it could be shown that they contained a keratan sulfate-rich region intercalated between the hyaluronate--binding region and a chondroitin sulfate-rich region. In nasal cartilage proteoglycans about 60 % of the keratan sulfate was found in the keratan sulfate-rich region, about 20 % in the chondroitin sulfate-rich region and 20 % in or close to the hyaluronate-binding region. The corresponding figures for chondroitin sulfate are approximately 10 %, 90 %, and 0 %. The hyaluronate-binding and the chondroitin sulfate-rich regions occupy each one third to half of the protein core while the keratan sulfate-rich region only occupies about one third of the protein core. The keratan sulfate peptides isolated from intervertebral disc proteoglycans are larger than the corresponding peptides from nasal and tracheal cartilage proteoglycans. Differences in the molecular size of the keratan sulfate chains may explain the observed variations in size.

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